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INHIBITION OF HUMAN RED CELL SODIUM AND POTASSIUM TRANSPORT BY DIVALENT CATIONS

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SUMMARY

1. The influx and efflux of Na and K across the human red cell membrane by the bumetanide-sensitive (Na–K co-transport) and residual (ouabain- and bumetanide-insensitive) routes were inhibited by increasing concentrations of external Mg.

2. Ca, Sr, Ba, Mn and Co also inhibited bumetanide-sensitive and residual K transport.

3. External Mg inhibited choline uptake and the Na-dependent fractions of L-alanine and L-serine uptakes.

4. External Mg reduced the maximal rate (app. V_{max}) but not the affinity (app. K_m) of the bumetanide-sensitive K and Na influxes when they were measured as functions of external K and Na respectively.

5. The inhibitory effect of Mg was not due to a small reduction in zeta potential since much larger reductions in zeta potential produced by neuraminidase did not affect transport.

6. Internal Mg stimulated the ouabain-sensitive K influx but inhibited the co-transport and residual components of K influx.

7. Bumetanide was a poor co-transport inhibitor in red cells pre-treated with A23187 and EDTA.

8. It was concluded that the inhibitory effects of external Mg were probably not due to changes in the ionic composition of the diffuse double layer adjacent to the cell membrane.

9. Mg and other divalent cations should not be used as 'inert' ionic substitutes in human red cell Na and K transport studies.

INTRODUCTION

Divalent cations in general, and Mg^{2+} in particular, have been frequently used as inert substitutes for Na^+ and K^+ in cation transport studies (Hoffman & Kregenow, 1966; Sachs, 1971; Dunn, 1973; see also discussion, Lew & Beaugé, 1979). This is perhaps surprising given the effects of divalent cations on the properties of excitable membranes and artificial lipid bilayers. Frankenhaeuser & Hodgkin (1957) showed that Ca^{2+} and Mg^{2+} stabilized the membrane of the squid giant axon against depolarization. One of their interpretations of this effect was that divalent cations

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adsorbed to the membrane created an electric field in the membrane that modified the transmembrane potential. The total electric field then influenced the gating mechanisms for ion movement through the membrane and hence the ion movement itself. This idea was elaborated on by others working with excitable tissues (for instance, Chandler, Hodgkin & Meves, 1965; Hille, Woodhull & Shapiro, 1975) and with phospholipid bilayers (McLaughlin, Szabo & Eisenman, 1971; McLaughlin, Grathwohl & McLaughlin, 1978). Their results can be summarized as follows. The cell membrane carries a net negative change due to the proteins and the head groups of the phospholipids. This negative charge increases the concentration of cations and decreases the concentration of anions in the diffuse double layer next to the membrane. Thus the conductance of the membrane and the transport of ions across it should depend on the magnitude of the surface charge. Divalent cations can reduce the negative charge by screening it or by binding to it or by a combination of these processes (see McLaughlin *et al.* 1971). They could therefore alter the conductance and transport characteristics of membranes.

The present study has been designed to examine the effects of Mg^{2+} and other divalent cations on the characteristics of several transport systems in human red cells and to see whether the diffuse double layer theory could explain the results. We have concentrated on three systems: (a) transport mediated by the Na pump which is measured by its sensitivity to inhibition by ouabain; (b) transport mediated by the Na–K co-transport system (see Wiley & Cooper, 1974) which is measured by its sensitivity to inhibition by bumetanide (the most potent co-transport system inhibitor amongst the commonly available loop diuretics, see Ellory & Stewart, 1982) or by removal of Cl^- (see Dunham, Stewart & Ellory, 1980; Chipperfield, 1980); (c) transport measured when both the Na pump and Na–K co-transport system have been inhibited. These are sometimes called the 'residual fluxes'. Residual K influx varies linearly with the concentration of external K and thus may represent passive diffusion through the membrane (Wiley & Cooper, 1974; Lew & Beaugé, 1979).

The presence on the red cell surface of an extensive glycoprotein complex containing acid sugar residues contributes a region of negatively charged groups at some distance from the phospholipid bilayer. It is these superficial sialic acid groups which principally contribute to the surface charge on red cells as measured from their electrokinetic behaviour, usually defined as the zeta potential (see Seaman, 1975). Seaman, Vassar & Kendall (1969) have shown that Ca^{2+} reduces the zeta potential of red cells while others have shown that Mg^{2+} can reduce transport in these cells (Rettori & Lenoir, 1972; Lew, Hardy & Ellory, 1973; Ellory, Flatman & Stewart, 1980). We have designed experiments to see whether there is a relationship between the reduction of zeta potential and the inhibition of transport by Mg^{2+}. We have also used neuraminidase to alter the zeta potential (Seaman, 1975) to see whether ion transport was affected and to see whether the responses of the transport systems to Mg^{2+} were altered. Finally, the effects of increasing Mg^{2+} concentration on organic cation (choline) transport, and charged and neutral amino acid uptake have been briefly investigated, to assess the specificity of the effect.

The results show that Mg^{2+} and other divalent cations do inhibit a wide variety of transport systems although it seems unlikely that the inhibition is simply due to changes in ion concentration in the diffuse double layer next to the membrane.

A brief account of this work had been published previously (Ellory *et al.* 1980).

METHODS

Solutions and materials

All solutions were prepared with double-glass-distilled water using Analar grade reagents (B.D.H.) where possible. Choline chloride was obtained from Sigma Ltd and was recrystallized from hot ethanol before use. Neuraminidase (type VII), ouabain, Triton X-100, MOPS, HEPES, EDTA, EGTA, bovine serum albumin and the amino acids were obtained from Sigma Ltd. Bumetanide was a gift from Leo Laboratories Ltd and A23187 was a gift from the Lilly Research Centre Ltd. All isotopes were obtained from the Radiochemical Centre, Amersham.

The experiments were carried out at 37 °C and pH 7·4 unless otherwise stated. All solutions contained 15 mM-MOPS, 5 mM-glucose and 150 mM-Cl and had a nominal osmolarity of 315 mosmol, unless otherwise stated. It was important to maintain the Cl concentration at 150 mM since the Na–K co-transport system is sensitive to changes in the Cl concentration (Dunham *et al.* 1980; Chipperfield, 1980). The sum of the concentrations of Na and K was normally 75 mM, the osmolarity and Cl concentration of the medium being maintained with sucrose and choline chloride. As the Mg concentration was increased from 0 to 37·5 mM, the ionic strength of the medium increased from 0·150 to 0·1875 mol/l.

Cells

Red cells from freshly drawn heparinized blood were washed three times by centrifugation and resuspension in at least ten volumes of isotonic saline (150 mM-NaCl, 15 mM-MOPS, 5 mM-glucose, pH 7·4 at 20 °C).

Replacement of cell Cl by NO_3 or methyl sulphate

In some experiments it was necessary to replace the Cl in the cells by NO_3 or methyl sulphate. The method is described by Dunham *et al.* 1980.

Alteration of cell Mg content

The concentration of internal ionized Mg ($[Mg^{2+}]_i$) was varied between $< 10^{-6}$ to $3{\cdot}4 \times 10^{-3}$ M by use of the ionophore A23187. The washed red cells were incubated at 37 °C, 10 % haematocrit and under constant magnetic stirring in media containing 145 mM-KCl, 5 mM-NaCl, 10 mM-HEPES (pH 7·7 at 37 °C), 0·1 mM-Tris–EGTA and varying concentrations of Mg. 3 μM-A23187 was then added to the suspension. This makes the membrane very permeable to Mg which leaves or enters the cells depending on the external Mg concentration (for detailed description see Flatman & Lew, 1980). After 15 min, the cells were washed free of A23187 by three washes with 50 vol. of the same incubation medium containing 1 mg/ml bovine serum albumin. (The details of the washing procedure are given in Flatman, 1982). The cells were finally washed with the relevant incubation medium to remove the albumin. The Mg content of the cells was measured by atomic absorption spectroscopy and the ionized Mg concentration was obtained from the red cell Mg buffering curve measured previously (Flatman & Lew, 1980).

Treatment of cells with neuraminidase

Washed red cells were incubated at 37 °C and 10 % haematocrit for 120 min with 20 μg/ml neuraminidase.

Na and K influx

Washed red cells were suspended at 3–5 % haematocrit in a total volume of 1 ml of medium contained in an Eppendorf 1·5 ml polypropylene microcentrifuge tube. 0·1 mM-ouabain and/or 0·1 mM-bumetanide were present as required. The tubes were incubated at 37 °C for 10 min after which time a small quantity of the relevant isotope was added (^{42}K or ^{86}Rb for K influx, ^{22}Na or ^{24}Na for Na influx), and the contents of the tubes were mixed. The incubations were stopped after 30 min by brief centrifugation at 10000 *g*, and the supernatants were removed by suction. The cells were then washed free of extracellular radioactivity by four successive suspensions and centrifugations in ice-cold isotonic $MgCl_2$ solution (108 mM-$MgCl_2$, 10 mM-Tris Cl, pH 7·4). The cell pellet was lysed with 0·5 ml of 0·1 % (v/v) Triton X-100 and the protein was precipitated by adding 0·5 ml of 5 % (w/v) trichloracetic acid followed by centrifugation at 10000 *g* for 2 min. The activity of ^{86}Rb, ^{42}K, or ^{24}Na in the supernatant was determined by Cerenkov counting in a Packard Tri-Carb scintillation counter. ^{22}Na activity was determined by liquid scintillation counting using Pico-fluor

30 scintillant (Packard Ltd). The specific activity of the labelled K or Na solutions was determined by counting the activity of a suitably diluted sample. The volume of red cells in the suspension was determined from the concentration of haemoglobin measured spectrophotometrically in a diluted sample after conversion to cyanomethaemoglobin. The conversion factor for relating optical density (O.D.) to haematocrit was O.D. = 250 is equivalent to packed cells (i.e. 100% haematocrit). This factor was independently determined from microhaematocrit measurements. All measurements were made in triplicate or quadruplicate and results were expressed as the mean ±S.E. of the mean. Influxes were expressed as mmol/l cells.h.

Na and K efflux

Washed red cells were loaded with ^{24}Na or ^{42}K by incubating them for 6 h at 37 °C at 10% haematocrit in a solution containing the isotope (1–2 mCi ^{24}Na; 200–300 μCi ^{42}K), 150 mM-NaCl or KCl, 15 mM-MOPS (pH 7·4 at 37 °C) and 10 mM-glucose. At the end of the incubation the cells were washed three times in ice-cold medium to remove external radioactivity. The final internal specific activity was in the range 0·1–0·2 mCi/mmol Na and 0·5–10 μCi/mmol K.

The cells were resuspended at 5% haematocrit in the appropriate media. 1 ml aliquots of each suspension were then quickly added to twelve 1·5 ml Eppendorf centrifuge tubes which were then incubated at 37 °C in a water-bath. At 10 or 20 min intervals three of the tubes were removed and centrifuged at 10000 *g* for 1 min. The β-activity in the supernatant was then measured in a Packard Tri-Carb β-counter. A suitably diluted sample of the whole suspension was also counted to assess the total activity in the cells initially. The efflux rate constants (k) were calculated from these data (see Garrahan & Glynn, 1967) using the method of least squares (see Draper & Smith, 1966). The results were expressed as $k \pm$S.D. (n = degrees of freedom).

Choline uptake

Washed cells were incubated for 3 h at 37 °C in media containing 75 mM-choline chloride (containing [methyl-^{14}C]choline chloride), 15 mM-MOPS, (pH 7·4 at 37 °C), 5 mM-glucose and either 50 mM-$MgCl_2$ or 75 mM-KCl. At the end of the incubation the cells were washed free of external radioactivity and the cells were processed and counted as for ^{22}Na influx (above).

Uptake of amino acids

The uptake of ^{14}C-labelled, L-leucine, L-lysine, L-valine, L-alanine and L-serine by red cells was examined using the methods of Young, Jones & Ellory (1980). The media contained: 75 mM-NaCl, 15 mM-MOPS (pH 7·4 at 37 °C), 5 mM-glucose, 0·2 mM of the relevant (^{14}C-labelled) amino acid and either 37·5 mM $MgCl_2$ + 37·5 mM-sucrose or 75 mM-choline chloride. The Na-dependent fractions of L-alanine and L-serine uptake were studied by substituting 75 mM-K.

Electrokinetic studies

The electrophoretic mobilities of control and neuraminidase-treated red cells were measured in media containing nominal 0 and 37·5 mM concentrations of Mg. The method is described by Bangham, Flemans, Heard & Seman (1958). The composition of the media was identical to that used for flux measurements. The field strength was approximately 3 V/cm.

Abbreviations

MOPS, 3-(N-morpholino) propanesulphonic acid; HEPES, N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

RESULTS

K influx

Fig. 1 shows the inhibition of ouabain-sensitive (Na–K pump), bumetanide-sensitive (Na–K co-transport) and residual (ouabain plus bumetanide-insensitive)) components of K influx as the concentration of external Mg was increased from zero (nominal) to 37·5 mM. All three components were significantly ($P < 0·01$) inhibited by 37·5 mM-Mg; the ouabain-sensitive and residual components being inhibited by 15%

and the bumetanide-sensitive component by 25 %. The experiments described above were carried out at constant nominal osmolarity (315 mosmol) and Cl concentration (150 mM). However, it was not possible to maintain the ionic strength at a constant level, and the ionic strength increased from 0·150 to 0·185 mol/l as the Mg concentration was increased from zero to 37·5 mM. Experiment I of Table 1 shows that if the Mg

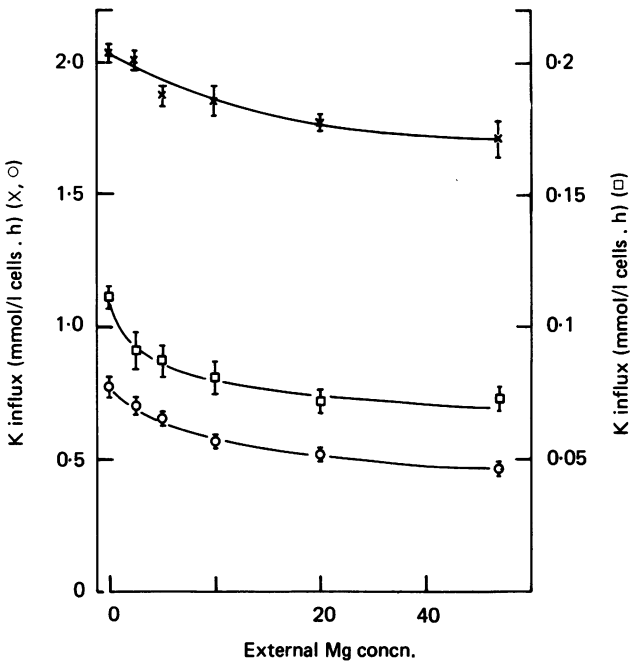

Fig. 1. Ouabain-sensitive (×), bumetanide-sensitive (○) and residual (□: note difference in units on abscissa) components of K influx as a function of the external Mg concentration. The solutions contained (mM): K, 7·5; Na, 67·5; Cl, 150; MOPS, 15 (pH 7·4 at 37 °C); glucose, 5; and ouabain and bumetanide, 0·1; as required. The concentration of Mg was varied between 0 and 37·5 mM, the osmolarity and Cl concentrations being maintained with sucrose and choline chloride. Each point represents the mean ±S.E. of the mean ($n = 3$).

concentration is increased from nominally zero to 25 mM at constant ionic strength and osmolarity (but with a small fall in chloride concentration) then all three modes of K influx are still significantly inhibited as before. This indicates that the inhibition seen in Fig. 1 is unlikely to be due to the increase in ionic strength with increasing Mg concentration. The inhibition of bumetanide-sensitive K influx in this experiment was greater than would be expected from the variation in Cl concentration (see Dunham *et al.* 1980, Fig. 2). It is possible, however, that some of the effects seen were due to changes in the concentration of choline chloride or sucrose used to maintain the Cl concentration and osmolarity. In Experiment II of Table 1 choline and sucrose were omitted, and the external Mg concentration varied between 0 and only 10 mM to prevent excessive changes in ionic strength. Even with this small increase in Mg concentration, both the bumetanide-sensitive and the residual components of K influx were significantly reduced ($P < 0·01$). The ouabain-sensitive component,

however, showed a small increase in size but this was not significant. This agrees with the finding of Flatman & Lew (1981), who showed that the efflux of Na through the Na pump was not affected by increasing the external Mg concentration up to 5 mM.

Since it was important to eliminate the possibility of external Mg modifying the interaction between the cells and ouabain or bumetanide, possibly reducing their effectiveness, control experiments were performed. The dose–response curve for ouabain inhibition (range 10^{-8}–10^{-5} M) of K influx was identical in the presence or absence of 25 mM-Mg in a Na medium. Also, [^{3}H]ouabain binding gave the same number of binding sites whether or not external Mg was present. For bumetanide,

TABLE 1. K influx under different ionic conditions

	Composition of medium (mM)						K influx (mmol/l cells.h) (K_o = 7·5 mM)		
Expt. no.	Mg	Na	Choline	Cl	Sucrose	Ionic strength (mol/l)	Ouabain-sensitive	Bumetanide-sensitive	Residual
I	0	75	75	150	0	0·150	1·589±0·032	0·841±0·017	0·118±0·006
	25	75	0	125	75	0·150	1·362±0·022	0·501±0·014	0·084±0·011
II	0	150	0	150	0	0·150	1·686±0·135	1·526±0·010	0·134±0·005
	10	135	0	155	0	0·185	1·805±0·024	1·084±0·009	0·117±0·002

The three components of K influx measured under different ionic conditions. All solutions contained the following: 15 mM-MOPS, 7·5 mM-glucose and the other constituents shown in the Table. pH was 7·4 and osmolarity was 315 mosmol. Results are expressed as the mean ± S.E. of mean (n = 3).

Cl substitution by NO_3 allowed a direct comparison of the bumetanide-sensitive and Cl-dependent components in Na and Mg media (Dunham *et al.* 1980). In 145 mM-Na, the bumetanide-sensitive K influx in 150 mM-Cl was 0·555±0·012 whilst the Cl-dependent component (NO_3 replacement) was 0·606±0·017 mmol/l cells.h. In 95 mM-Na, 25 mM-$MgCl_2$, 25 mM-sucrose, these fluxes were 0·342±0·010 and 0·380±0·011 respectively. We therefore conclude that external Mg does not interfere with the action of either of these two inhibitors (but compare with internal Mg, see below).

The effectiveness of other divalent cations in inhibiting the bumetanide-sensitive component of K influx is shown in Fig. 2. Each divalent cation significantly inhibited the flux (errors being omitted from Fig. 2 for clarity), though there were small differences in potency.

Na influx

Fig. 3 shows the effects of increasing the external Mg concentration on the bumetanide-sensitive and residual (bumetanide- and ouabain-insensitive) components of Na influx. 37·5 mM-Mg inhibited the bumetanide-sensitive component (Na–K co-transport) by 40 % and the residual component by 35 %. Table 2 shows the effects of 37·5 mM-Ca or Ba on Na influx. Ca and Ba were slightly more potent at inhibiting bumetanide-sensitive Na influx than Mg (50 and 53 % inhibition by 37·5 mM-Ca and Ba respectively). Ca was as effective, and Ba was less effective than Mg at inhibiting the residual Na influx (33 % and 18 % inhibition by 37·5 mM-Ca and Ba respectively).

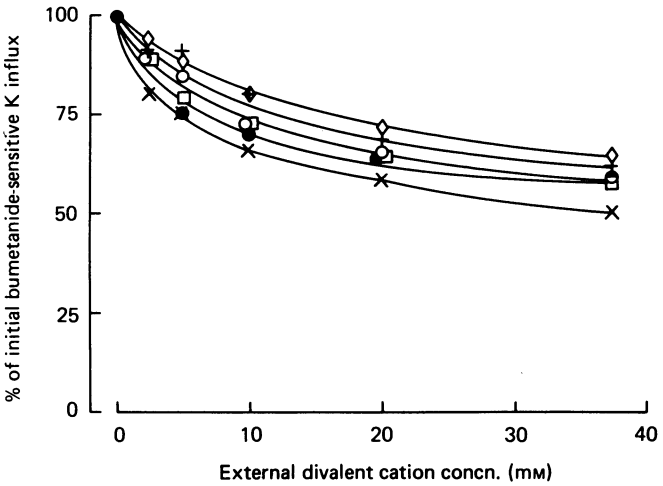

Fig. 2. Bumetanide-sensitive K influx as a function of external divalent cation concentration. The conditions were identical to those specified for Fig. 1, except that Sr, ◇; Ba, +; Ca, □; Mn, ● and Co, ×; were substituted for Mg, ○. Curves drawn by eye.

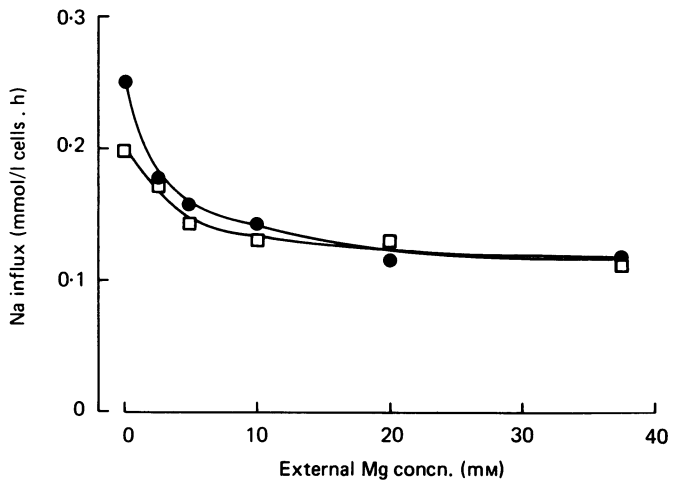

Fig. 3. Bumetanide-sensitive (●) and residual (□) components of Na influx as a function of external Mg concentration. The solutions were similar to those described in Fig. 1 except that the concentrations of Na and K were 7·5 and 67·5 mM respectively. The points represent the mean of three determinations. The S.E. of the means were within the limits of the points.

K and Na efflux

Fig. 4 shows that increasing the concentration of external Mg from 0 to 10 mM inhibited both the bumetamide-sensitive and residual components of K efflux. This inhibition is similar to the inhibition of Na and K influx described above.

Fig. 5 shows the effects of increasing the external Mg concentration on ouabain-sensitive, bumetanide-sensitive and residual components of Na efflux. The effects of between 0 and 37·5 mM-Mg are shown in panel *A*. In this experiment the concentration of external Na was 50 mM and isotonicity was maintained with sucrose and choline chloride as the concentration of Mg was altered. All three components of Na efflux

TABLE 2. Na influx in external Ca and Ba media

Media composition (mM)			Na influx (mmol/l cells.h)	
Choline	Ca	Ba	Bumetanide-sensitive	Residual
75	—	—	0·220±0·008	0·165±0·005
—	37·5	—	0·109±0·004	0·110±0·004
—	—	37·5	0·103±0·019	0·135±0·006

Na influx measured in the presence of Ca or Ba. The solutions were the same as those described for Fig. 3 except that Ca and Ba were used in place of Mg. The results are expressed as the mean±S.E. of mean ($n = 3$).

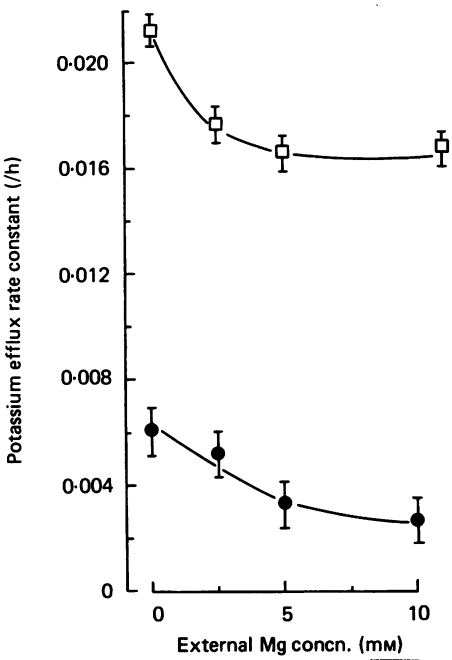

Fig. 4. Bumetanide-sensitive (●) and residual (□) components of K efflux as a function of external Mg concentration. The solutions contained (mM): K, 25; Na, 50; Cl, 150; MOPS, 15 (pH 7·4 at 37 °C); glucose, 5; ouabain 0·1; Mg, 0–10, and bumetanide, 0·1 if required. Sucrose and choline were used to maintain osmolarity. The error bars denote the S.E. of the rate constant.

were inhibited by Mg. An experiment was also carried out at a higher Na concentration (125 mM) and in the absence of sucrose and choline to see whether transport was still affected by Mg. In this experiment the concentration of Mg was only increased to 5 mM to prevent excessive changes in osmolarity, ionic strength and chloride concentration. Panel *B* shows that under these conditions, the bumetanide-sensitive and residual fluxes were significantly inhibited ($P < 0·01$), but there was no significant ouabain-sensitive component inhibition, consistent with the data shown in Table 1 and with the findings of Flatman & Lew, 1981.

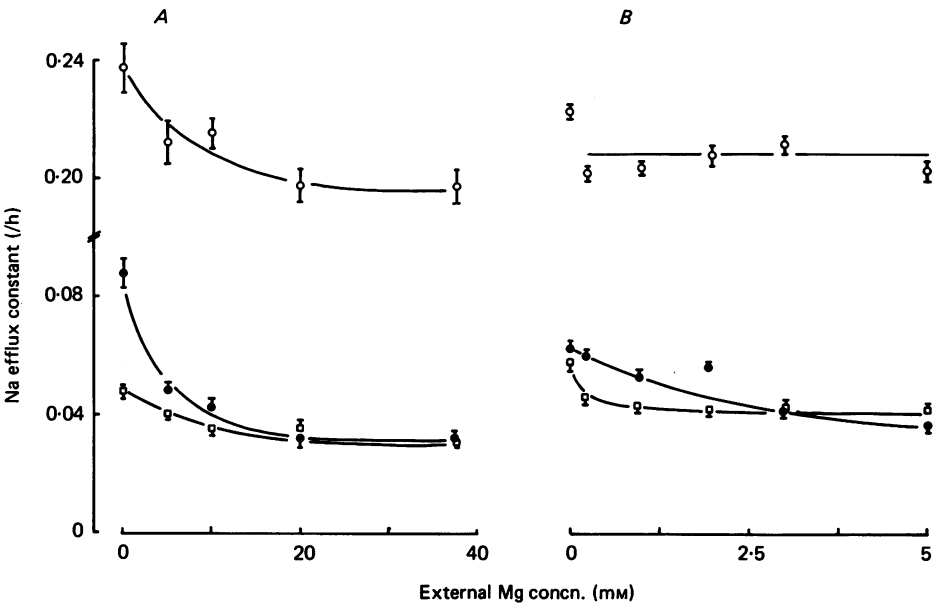

Fig. 5. Ouabain-sensitive (○), bumetanide-sensitive (●) and residual (□) components of Na efflux as functions of external Mg, over the ranges 0–37·5 mM (panel *A*) and 0–5 mM (panel *B*). In the experiment shown in panel *A*, the media contained (mM): Na, 50; K, 25; Cl, 150; MOPS, 15 (pH 7·4 at 37 °C); glucose, 5; and ouabain or bumetanide 0·1, as required. The concentration of Mg was varied between 0 and 37·5 mM, osmolarity and Cl concentration being maintained with sucrose and choline chloride. In the second experiment (panel *B*) the concentrations of MOPS, K, glucose, and inhibitors were the same, as in panel *A*, the concentration of Na was 125 mM, and $MgCl_2$ was added as shown. Results are expressed as rate constant k (/h) ± S.D. where $n = 8$.

Effects of external Mg on kinetics of bumetanide-sensitive Na and K influx

One possible explanation of the inhibition of ion transport by divalent cations is that they reduce the surface potential of the membrane. This would result in a change in the concentration of ions at the surface of the membrane where the ion binding sites are situated. Thus divalent cations might be expected to increase the app. K_m of carrier-mediated cation transporters. We investigated this prediction for the Na–K co-transport system by measuring the dependence of bumetanide-sensitive K and Na influx on external K and Na respectively. Fig. 6 shows the effect of 25 mM-external Mg on the K dependence of bumetanide-sensitive K influx under conditions of constant Cl concentration and osmolarity. The K dependence of K influx could be described by Michaelis–Menten kinetics in both cases. In the absence of Mg, the V_{max} was 1·43 ± 0·04 mmol/l cells . h and the K_m was 6·58 ± 0·83 mM. In the presence of 25 mM-Mg, the V_{max} fell by 35% to 0·88 ± 0·08 mmol/l cells . h but the K_m was not significantly changed at 7·38 ± 0·74 mM. A similar pattern was seen with the Na dependence of Na influx (Fig. 7): 25 mM-Mg caused a reduction of 40% in V_{max} (from 0·52 ± 0·07 to 0·30 ± 0·06 mmol/l cells . h, whereas the K_m was not significantly altered (from 18·6 ± 2·2 to 17·6 ± 1·4 mM).

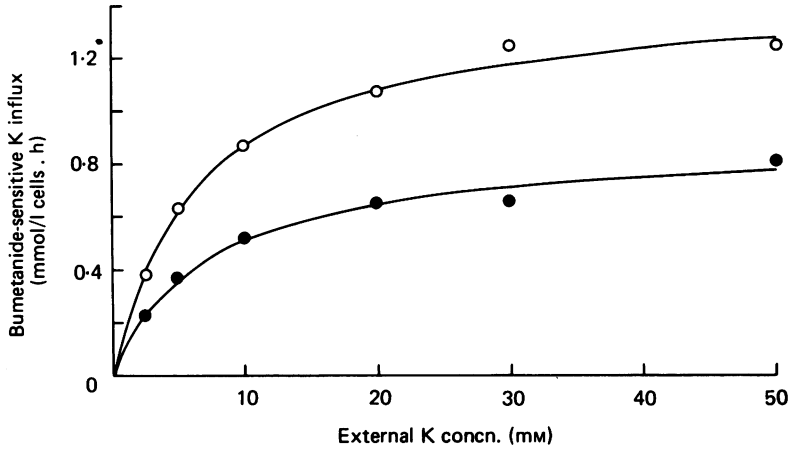

Fig. 6. Bumetanide-sensitive K influx as a function of external K in the presence (●) and absence (○) of 25 mM-external Mg. The media contained (mM): Na, 50; MOPS, 15; glucose, 5; ouabain, 0·1; bumetanide, 0·1 as required; Mg, 25; as required, and between 2·5 and 50 K. Sucrose and choline chloride were used to maintain osmolarity at 315 mosmol and Cl concentration at 150 mM. The points represent the means of three separate determinations and the S.E. of the mean lies within the symbol size. The data was redrawn as a double reciprocal plot and the K_m and V_{max} were calculated by linear-regression analysis (Draper & Smith, 1966). The lines through the data shown above were then drawn according to the equations:

$$\text{K influx in absence of Mg} = \frac{1{\cdot}43 \times [K]_o}{6{\cdot}58 + [K]_o} \text{ mmol/l cells . h.}$$

$$\text{K influx in presence of 25 mM-Mg} = \frac{0{\cdot}88 \times [K]_o}{7{\cdot}38 + [K]_o} \text{ mmol/l cells . h,}$$

where $[K]_o$ is the concentration of external K (mM).

Effects of internal Mg on Na–K co-transport

Having explored the effects of changing external Mg on ion transport we decided to examine the effects of internal Mg to see whether the effects were symmetrical. The internal Mg content of cells was altered using the ionophore A23187 (see Flatman & Lew, 1980), which was then washed away leaving the cell membrane with its usual very low Mg permeability. For initial experiments two types of cell were produced: one group had a very low internal ionized Mg concentration ($< 10^{-6}$ M) while the other contained about 1 mM-ionized Mg. Table 3 shows that when the cells contained 1 mM-ionized Mg the ouabain-insensitive K influxes were similar to those in control cells, and that bumetanide inhibited about 80–90 % of these fluxes. The residual fluxes seen in the presence of bumetanide and ouabain (0·06–0·11 mmol/l cells . h) were similar to residual fluxes in control cells (see Table 1 or Fig. 1). The normal ionized Mg concentration in oxygenated red cells is about 0·4 mM (Flatman, 1980), thus more than doubling the normal internal ionized Mg does not drastically alter the behaviour of the ouabain-insensitive K-transport systems. However, when the ionized Mg inside the cells was reduced to very low levels ($< 10^{-6}$ M) the bumetanide-sensitive component fell to about half its normal level whereas the total ouabain-insensitive flux was not affected. These experiments were carried out with 2 mM-EDTA in the medium to make sure that no Mg could enter the cells. However, control experiments where 1 mM-Mg was present in the medium gave exactly the same picture. These

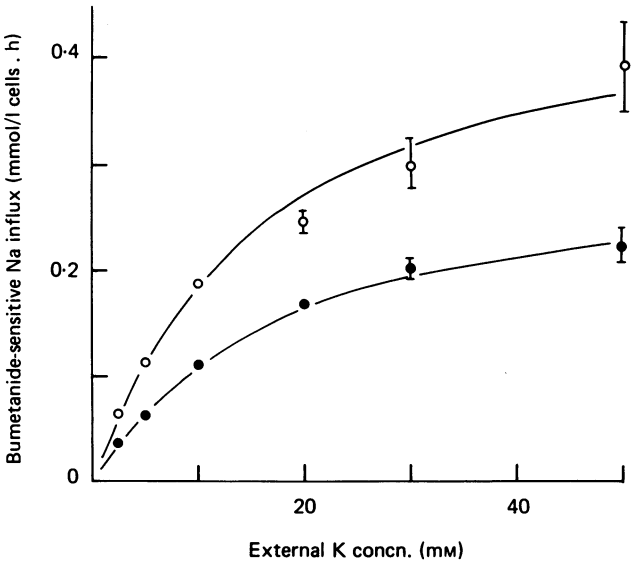

Fig. 7. Bumetanide-sensitive Na influx as a function of external Na in the presence (●) and absence (○) of 25 mM-Mg. The media contained (mM): K, 50; MOPS (pH 7·4), 15; glucose, 5; ouabain, 0·1; bumetanide, 0·1 as necessary; Mg, 25 as necessary and between 2·5 and 50 mM-Na. Sucrose and choline chloride were used to maintain the osmolarity at 315 mosmol and Cl concentration of 150 mM. The points are the mean ± S.E. of the mean ($n = 3$). The data were redrawn as a double reciprocal plot, and the K_m and V_{max} were calculated by linear-regression analysis. The lines through the data shown above were then drawn according to the equations:

$$\text{Na influx in the absence of Mg} = \frac{0{\cdot}52 \times [\text{Na}]_o}{18{\cdot}6 + [\text{Na}]_o} \text{ mmol/l cells . h,}$$

$$\text{Na influx in presence of 25 mM-Mg} = \frac{0{\cdot}30 \times [\text{Na}]_o}{17{\cdot}6 + [\text{Na}]_o} \text{ mmol/l cells . h,}$$

where $[\text{Na}]_o$ is the external Na concentration.

TABLE 3. Effect of 0·1 mM-bumetanide on cells pre-treated with A23187 and either EDTA or Mg

	Magnesium/ concentration (mM)			Ouabain-insensitive K influx (mmol/l cells . h)		
Expt. no.	Internal	External	Anion	Total	With 0·1 mM-bumetanide	Bumetanide-sensitive
1	$< 10^{-3}$	1	Cl	0·664 ± 0·015	0·342 ± 0·017	0·321 ± 0·023
2	1	1	Cl	0·662 ± 0·016	0·062 ± 0·005	0·600 ± 0·017
3	$< 10^{-3}$	0*	Cl	0·724 ± 0·015	0·447 ± 0·014	0·277 ± 0·021
4	1	0*	Cl	0·724 ± 0·037	0·106 ± 0·003	0·618 ± 0·037
5	$< 10^{-3}$	1	NO_3	0·105 ± 0·004	—	—
6	1	1	NO_3	0·101 ± 0·003	—	—
7	$< 10^{-3}$	0*	NO_3	0·146 ± 0·007	—	—
8	1	0*	NO_3	0·117 ± 0·003	—	—

* 2 mM EDTA in medium during flux.

The effects of internal and external ionized Mg on the inhibition of K influx by bumetanide or ›y replacement of Cl by NO_3. The media contained (mM): KCl, 7·5; NaCl, 142·5; MOPS (pH 7·4), .5; glucose, 5; ouabain 0·1 and Mg, 1 or EDTA, 2. The Mg content of the cells was altered using \23187 which was washed away before the start of the flux measurement. The cells contained either $< 10^{-6}$ M or 10^{-3} M ionized Mg (see Methods). K influx was measured using ^{86}Rb as a tracer. Results ıre given as the mean ± S.E. of mean ($n = 3$).

results suggest that internal Mg may be required for bumetanide to completely inhibit the Na–K co-transport rather than that the co-transport system itself is internal Mg-dependent. In order to test this hypothesis, the Cl inside and outside the cells was replaced by NO_3. This procedure results in inhibition of Na–K co-transport which is Cl-dependent (see Dunham *et al.* 1980; Chipperfield, 1980). Table 3 shows that, at both low and high internal Mg concentrations, K influx was reduced by 80–90% of that seen in control cells. The remaining K influxes were similar to the residual fluxes seen in normal cells treated with ouabain and bumetanide. It therefore appears that internal Mg may be required for effective inhibition of Na–K co-transport by bumetanide.

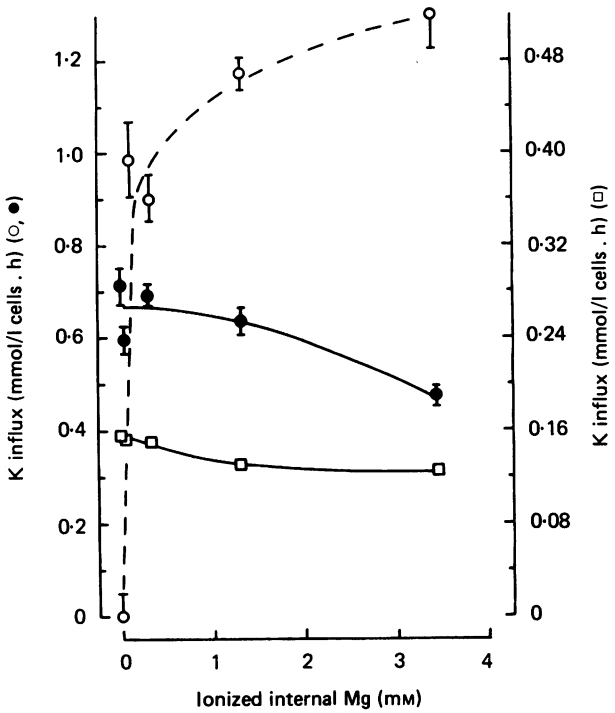

Fig. 8. K influx as a function of internal ionized Mg concentration. The internal ionized Mg concentration was altered using A23187 as described in the Methods. The A23187 was washed away before the start of the flux measurements. The flux media contained (mM): K, 7·5; Na, 142·5; MOPS, 15; glucose, 5; ouabain, 0·1 as required and Cl or methylsulphate, 150. K influx was measured using ^{86}Rb as a tracer. The components of K influx are shown as follows: ○---○, ouabain-sensitive; ●–● ouabain-insensitive, Cl-dependent; □–□ ouabain-insensitive, chloride-independent (residual). The points represent the mean ± S.E. of the mean of three determinations. The lines through the points were drawn by eye. The ionized Mg concentration in normal oxygenated red cells is about 0·4 mM (Flatman, 1980).

Having established that bumetanide was not an effective blocker of Na–K co-transport at low internal Mg concentrations, it was decided to examine the internal Mg dependence of K influx through the Na–K co-transport by anion substitution experiments (replacing Cl by methyl sulphate in this case). Fig. 8 shows the internal Mg dependence of the ouabain-sensitive, ouabain-insensitive Cl-dependent, and ouabain-insensitive Cl-independent (residual) components of K influx. It can be seen that ouabain-sensitive K influx was negligible at very low Mg levels (less than 10^{-6} M),

but that it was markedly stimulated as the Mg level rose. This is similar to the stimulation of ouabain-sensitive Na efflux by internal Mg (Flatman & Lew, 1981) and reflects the Mg dependence of the Na pump. The two passive components of K influx showed a downward trend as internal Mg was raised in a manner similar to that described for external Mg.

Choline uptake

The presence of 50 mM-external Mg (K replacement) reduced choline uptake (at 75 mM) from 0·183 ± 0·004 to 0·146 ± 0·001 mmol/l cells . h, a decrease of 20 %, which is significant and consistent with the inhibition of Na and K influx by external Mg.

Amino-acid transport studies

Table 4 shows that 37·5 mM-external Mg inhibits the Na-dependent uptake of L-alanine and L-serine via the ASC system (small neutral amino acid transport system [alanine, serine, cysteine] Young *et al.* 1980) by 40 %. Mg did not, however, affect the Na-independent uptake of L-alanine or L-serine and nor did it affect the uptake of L-leucine or L-valine, amino acids whose transport is independent of Na and occurs principally on the L system (neutral amino acid transport system) (see Young *et al.* 1980). Interestingly, transport of the cationic amino acid lysine via the ly^+ system was not inhibited by Mg^{2+} (in fact the flux showed a slight increase, Table 4).

TABLE 4. Amino-acid uptake in presence and absence of 37·5 mM-external Mg

	Composition of medium (mM)				Amino-acid uptake (mmol/l cells . h)	
Amino acid	Na	K	Choline	Mg	Total	Na-dependent fraction
Alanine	75	—	75	—	0·406 ± 0·016	0·228 ± 0·017
	—	75	75	—	0·178 ± 0·007	
	75	—	—	37·5	0·312 ± 0·018	0·131 ± 0·018
	—	75	—	37·5	0·181 ± 0·004	
Serine	75	—	75	—	0·0844 ± 0·0004	0·0688 ± 0·0013
	—	75	75	—	0·0156 ± 0·0012	
	75	—	—	37·5	0·0545 ± 0·0004	0·0398 ± 0·0006
	—	75	—	37·5	0·0147 ± 0·0004	
Lysine	75	—	75	—	1·035 ± 0·016	
	75	—	—	37·5	1·139 ± 0·017	
L-leucine	75	—	75	—	4·16 ± 0·12	
	75	—	—	37·5	4·04 ± 0·14	
Valine	75	—	75	—	2·10 ± 0·13	
	75	—	—	37·5	2·16 ± 0·05	

Amino-acid uptake in the presence and absence of 37·5 mM-Mg. The media contained (mM): NaCl, 75; MOPS (pH 7·4), 15; glucose, 5; 0·2 mM of the relevant ^{14}C-labelled amino acid and either choline chloride, 75 or sucrose, 37·5 and Mg, 37·5. Na-dependent fluxes were measured by replacing external Na by K. Results are expressed as the mean ± S.E. of mean for three determinations.

Electrokinetic studies

Experiments were designed to examine the relationship between the zeta potential and ion transport (K influx). The zeta potential was altered with Mg and by treatment of the cell with neuraminidase which cleaves negatively charged sialic acid residues from the cell surface (e.g. see Seaman, 1975).

Table 5 shows that while neuraminidase treatment rendered the cells isoelectric ($|\zeta| < 4$ mV) the bumetanide-sensitive and residual components of K influx measured in these cells were not significantly affected. If 37·5 mM-Mg was added to the medium containing control cells, both components of K influx were significantly reduced and the zeta potential increased by 4·4 mV. The addition of 37·5 mM-Mg to the neuraminidase-treated cells also caused a significant reduction in both components of K influx and the cells remained isoelectric. We conclude that there is little correlation between changes in zeta potential and the inhibition of ion transport.

TABLE 5. The effect of external Mg and neuraminidase treatment on the electrokinetic potential, and K influx in human red cells

Expt. no.	Cell pre-treatment	Composition of the media (mM)		K influx (mmol/l cells.h)		Electrokinetic potential (mV)
		Choline	Mg	Bumetanide-sensitive	Residual	
I	Control	75	—	0·60±0·03	0·14±0·02	−13·4±0·2
II	Neuraminidase	75	—	0·67±0·01	0·11±0·01	Isoelectric
III	Control	—	37·5	0·37±0·01	0·06±0·01	−9·0±0·2
IV	Neuraminidase	—	37·5	0·36±0·01	0·05±0·01	Isoelectric

Electrokinetic mobility compared with K influx. All solutions contained (mM): KCl, 7·5; NaCl, 67·5; MOPS (pH 7·4), 15; glucose, 5; ouabain and bumetanide, 0·1 as required and $MgCl_2$, 37·5 as indicated. Sucrose and choline chloride were used to maintain the osmolarity and Cl concentration (150 mM). Cells treated with neuraminidase had been exposed to 20 ng/ml neuraminidase prior to flux and electrokinetic measurements (see Methods). Results are given as the mean±S.E. of the mean for three determinations of the fluxes and twelve determinations of electrokinetic mobility.

DISCUSSION

The original purpose of these experiments was to investigate the effects of external divalent cations on Na and K transport. Specifically, we have examined the bidirectional fluxes of Na and K and the influxes of some non-electrolytes across the human red cell membrane in the presence of varying concentrations of external Mg. Cation fluxes were dissected into Na pump, Na–K co-transport and residual components using the two inhibitors ouabain and bumetanide. Control experiments ruled out an interaction between drug binding and the external presence of cations. Facilitated (bumetanide sensitive) and residual 'diffusional' components of cation transport were clearly inhibited by increasing external Mg concentrations in media of either low or high ionic strength (sucrose with choline or NaCl replacement). External Mg also inhibited Na–K efflux via these pathways. The Na–K-pump-mediated fluxes were also inhibited but only in the sucrose medium with low external Na (50–70 mM). The transport of two other cationic species was studied: choline uptake which was inhibited by external Mg, and the influx of the positively charged amino acid lysine where Mg slightly stimulated the flux. A further example of Mg inhibition of an Na-dependent membrane transport system was the marked decrease in amino acid uptake (alanine and serine) via the ASC system (Young *et al.* 1980). In this context the Na-independent fluxes of alanine, serine, and valine were not significantly affected.

For cation transport, the inhibition was not a specific effect of Mg but was a general

property of divalent cations. Ca, Ba, Sr, Co and Mn all inhibited co-transport K influx to about the same extent. In more limited studies Ca and Ba were also effective inhibitors of the bumetanide-sensitive and the residual Na influx. Internal Mg was also shown to inhibit transport. Increasing the concentration of internal ionized Mg up to 4 mM resulted in the inhibition of the co-transport (defined as Cl-sensitive) and residual (defined as Cl-insensitive) components of ouabain-insensitive K influx. Ouabain-sensitive influx was stimulated by internal Mg, consistent with the known effects of internal Mg on the Na–K pump (see Glynn & Karlish, 1975; Flatman & Lew, 1981). The experiments which varied internal Mg suggested a *trans* membrane effect consistent with the inhibitory effects of external Mg on K efflux.

An obvious approach to the interpretation of this divalent cation effect is to consider it in terms of varying electrostatic surface potential. The red cell external surface carries net negative charge principally in the form of acidic sugars, although there is a contribution from acidic phospholipid head groups and glycoproteins (Gahmberg, 1981). This surface charge results from a negative surface potential which in turn influences the concentration of ions in the medium immediately adjacent to the surface (Aveyard & Haydon, 1973). In line with theories in agreement with experiments for excitable tissue (e.g. see Hille *et al.* 1973) and artificial bilayers (e.g. McLaughlin *et al.* 1971), it might be suggested that in the studies described here, the divalent ions are neutralizing or screening negative surface charges, rendering the surface potential less negative, which could in turn reduce the transport rate for cations. In addition, it might be expected that there would be a decrease in affinity of a saturable carrier for external cation, since in the presence of the divalent cation greater bulk concentration of univalent cation would be required to maintain the appropriate surface concentration of univalent cation necessary for half-maximal activation of the carrier. No such effect was seen for the Na–K co-transport system where the Mg inhibition was a V_{max} and not a K_m effect (Figs. 6 and 7). Also, the surface concentration of not only univalent cations but also that of the positively charged amino acid lysine would be expected to be influenced by the divalent cation; the influx of lysine was, if anything, slightly stimulated in the Mg medium. The influx of choline was, however, reduced in the Mg medium. In a further series of experiments red cells were treated with neuraminidase, dramatically altering the electrokinetic potential. However, K-influx experiments on these cells failed to detect significant inhibition. This contrasted with the relatively small change in electrokinetic potential in the Mg-treated cells. It should be noted however that these electrokinetic measurements reflect only the potential at the plane of fluid shear between the cell and the medium, which may be some distance from the membrane surface proper (Seaman, 1975).

The interaction between divalent cations and phospholipid head groups is not solely confined to variations in surface potential. Studies of lipid bilayer ordering show that, in general, divalent cations can somehow 'stabilize' many types of phospholipid bilayer such that, in the presence of the divalents, the acyl chains tend to undergo the transition from liquid to gel phases at higher temperatures. Such effects have been demonstrated for phosphatidylethanolamine/phosphatidylserine mixtures by Tilcock & Cullis (1981), using ^{31}P n.m.r. (nuclear magnetic resonance); by Onishi & Ito (1974) for phosphatidylserine/phosphatidylcholine bilayers by electron spin resonance

(where Ca but not Mg was effective); by Trauble & Eibl (1974) for a number of charged phospholipids using a fluorescent probe; by Jacobson & Papahadjopoulos (1975) for phosphatidylcholine and phosphatidic acid during differential scanning calorimetry; and by Ligeti & Horvath (1980) for rat liver mitochondrial membranes using spin-labelled lipids.

It is possible that in the experiments described here, divalent-cation-induced membrane ordering, possibly secondary to an interaction between the phospholipid head groups and the divalents, is leading to an alteration in the packing of the chains and a generalized reduction in bidirectional univalent cation transport.

Whatever the precise mechanism, it seems clear that divalent cations should not be employed as supposedly 'inert' ionic substitutes in human red cell transport studies involving Na and K.

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